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14. ABSTRACT
The overall goal of this proposal is to test targeted carbon nanotubes for their ability to thermally ablate kidney cancer. Carbon nanotubes (CNTs) have been shown to be efficient transducers of near-infrared radiation for laser-induced thermal therapy of kidney cancer in animal models. However, the current generation of carbon nanotubes lacks the ability to selectively target cancer cells following systemic administration. In this proposal, we will develop carbon nanotubes designed to bind to uPAR, a surface receptor overexpressed in kidney cancers and supporting endothelium that is involved in growth, migration, proliferation, metastasis and angiogenesis. To date, we have demonstrated the overexpression of uPAR in kidney cancer cells and endothelial cells. We have shown that these cells are sensitive to nanotube-mediated thermal ablation. We have begun the synthetic steps required to create a nanotube conjugate with the targeting ligand. This grant is a mentor/pre-doctoral award which also focuses on training of the predoctoral candidate. In addition to carrying out the bench research described above, the trainee has also attended several national meetings on nanotechnology sponsored by the NCI and AACR. In the upcoming year, our plans are to complete the synthesis of nanoconjugates, explore their properties in vitro, and begin an examination of their properties in vitro.

15. SUBJECT TERMS
nanotube/thermal ablation/kidney cancer

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INTRODUCTION

The overall goal of this proposal is to test targeted carbon nanotubes for their ability to thermally ablate kidney cancer. Carbon nanotubes (CNTs) have been shown to be efficient transducers of near-infrared radiation for laser-induced thermal therapy of kidney cancer in animal models. However, the current generation of carbon nanotubes lacks the ability to selectively target cancer cells following systemic administration. In this proposal, we will develop carbon nanotubes designed to bind to uPAR, a surface receptor overexpressed in kidney cancers that is involved in growth, migration, proliferation, metastasis and angiogenesis. Binding of peptide fragments of kininogen (D5) to uPAR induces apoptosis in endothelial cells and inhibits tumor growth and metastasis. We will combine the anti-tumor properties of CNTs with those of D5 into one combined treatment by conjugating D5 peptides to several types of multiwall carbon nanotubes. *In vitro* experiments will be performed to test the specificity of binding of these conjugates to uPAR in human endothelial cells, kidney cancer cells, and kidney cells. Thermoablative properties, proliferation, survival, apoptosis, and downstream signaling of uPAR will be examined in these cell lines following treatment with D5 nanotubes. The anti-tumorigenic effect of these nanotubes will also be studied *in vivo*. Biodistribution, accumulation, and thermoablation will be studied initially. Human kidney cancer cells will be injected into the kidney capsule of nude mice. The effect of D5 nanotube injection and thermoablation on tumor growth and survival will be determined. The effect of D5 nanotubes on tumor angiogenesis will be studied by repeating these experiments using co-injection of human kidney cancer cells with human endothelial cells in a matrigel plug. We believe that these conjugated nanotubes will be able to demonstrate enhanced tumor ablation via targeting compared to thermal ablation alone.

BODY

Specific Aim 1. Synthesize nanotube-based particles ligated to D5s.

Progress on the Tasks from the Statement of Work associated with accomplishing this Specific Aim is described below.

Task 1: Obtain linear, Y-branched and dendritic nanoparticles.
The first batch of Y branched nanotube structures were fabricated by our collaborators at Rice University and have been sent to our laboratory (Task 1A). We have also received linear nanotubes (Task 1C). The experiments described below are being carried out with these linear nanotubes.

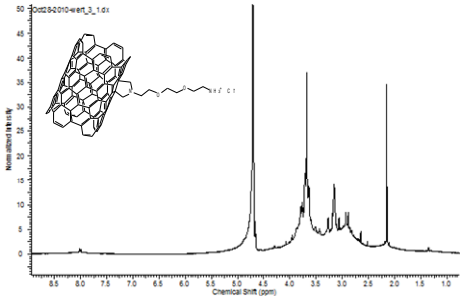

Figure 1NMR spectrum of PEG-primary amine MWCNT prepared in our laboratory

Task 2: conjugate linear, Y-branched and dendritic nanotubes to the synthetic peptide, D5.
We are in the process of optimizing conjugation methodology (Tasks 2a and 2b). Our collaborator, Dr. Bruce King, has been spearheading this facet of the project. We used the

Prato reaction [1], which generates a modified nanotube with a free primary amine group, and has been previously used for functionalization of nanotubes with peptides, proteins, and nucleic acids. To date, we have synthesized and characterized the well-known water soluble PEG-primary amine MWCNT using this method (Fig. 1).

Our next step is to couple this primary amine-derived MWCNT to D5, using an activated D5 carboxylic acid derivative. Specifically, amine bearing MWCNTs and nanoribbons will be coupled to N-terminal and side chain protected peptides through carboxylic acid activation using *O*-(7 aza-*N*-hydroxybenzotriazol-1-yl)-1, 1, 1, 3-tetramethyluronium hexafluorophosphate (HATU) and diisopropyl ethylamine (DIPEA) in DMF as reported for other peptide-carbon nanotube conjugates.

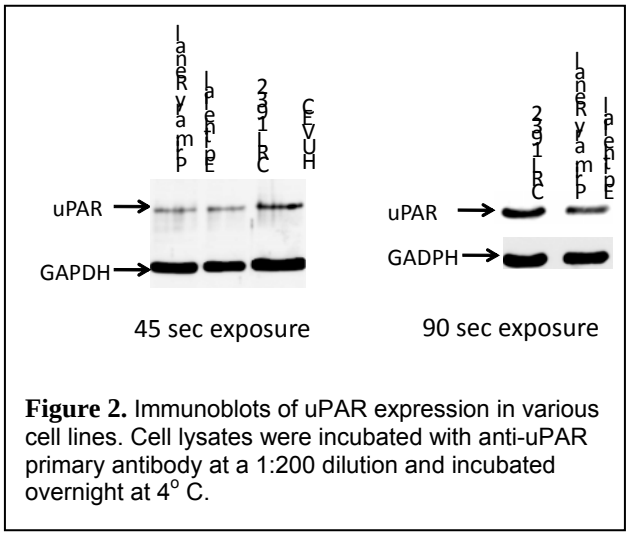

Figure 2. Immunoblots of uPAR expression in various cell lines. Cell lysates were incubated with anti-uPAR primary antibody at a 1:200 dilution and incubated overnight at 4° C.

Specific Aim 2. Test binding, cytotoxic and thermoablative properties of D5s-nanoparticles *in vitro*.

2.a. Test specificity of binding of D5a-nanoparticles.

Progress on the Tasks from the Statement of Work associated with accomplishing this Specific Aim is described below.

Task 2:1-2.3: Confirm that binding occurs via specific binding to UPAR and test ability of D5s-nanoparticles to selectively bind proliferating endothelial cells.

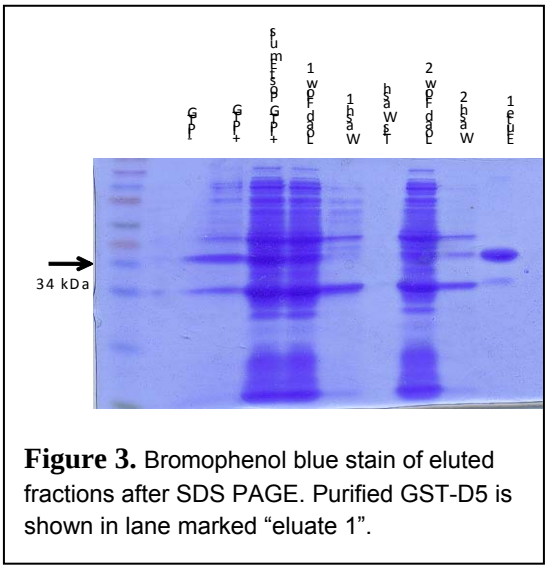

Figure 3. Bromophenol blue stain of eluted fractions after SDS PAGE. Purified GST-D5 is shown in lane marked “eluate 1”.

UPAR expression. We first confirmed that uPAR, the receptor targeted by D5, is expressed in renal cancer and endothelial cells, since these are the cells we plan to target with our D5-nanotube conjugate.

Protein expression of uPAR was assessed in the following cell lines by immunoblotting:

- HUVEC (human umbilical vein endothelial cells)
- Mixed Human Primary Renal Epithelial Cells
- CRL 1932 (human renal clear cell adenocarcinoma)
- RENCA (mouse renal adenocarcinoma)

Figure 2 shows increased expression of uPAR in HUVECs compared to normal and cancer cells and increased uPAR expression in cancer cells compared to normal cells.

Recombinant D5 Production. We also produced recombinant D5 for ligation to MWCNT. The full domain 5 of HKa (with a GST tag) was recombinantly expressed in chemically competent bacteria, harvested and purified using a Ni-NTA agarose column. GST-D5 is 34 kDa in size (**Figure 3**).

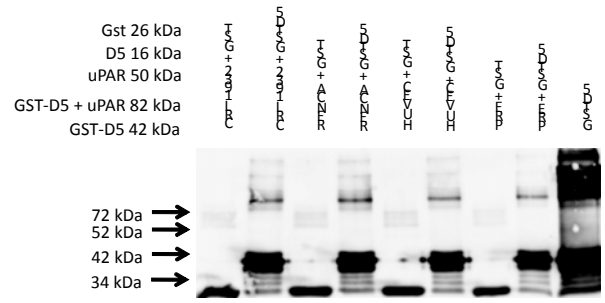

Figure 4. Immunoblot of GST pulldown of uPAR using cell lysates. Membrane was incubated with anti-GST antibody at 1:10,000 dilution for 1 hr.

D5-uPAR Binding. To test binding of D5 to uPAR, GST pulldown experiments were performed using cell lysates and GST-tagged D5. 20 ug of each cell lysate were incubated with GST-D5 or GST and rotated overnight at 4^{o} C. The lysates were then incubated with GST-binding agarose beads, washed, eluted, and ran on SDS PAGE. Binding was detected by immunoblotting for GST. (**Figure 4**) The pulldown experiment was also repeated using just CRL1932 lysate. **(Figure 5**)

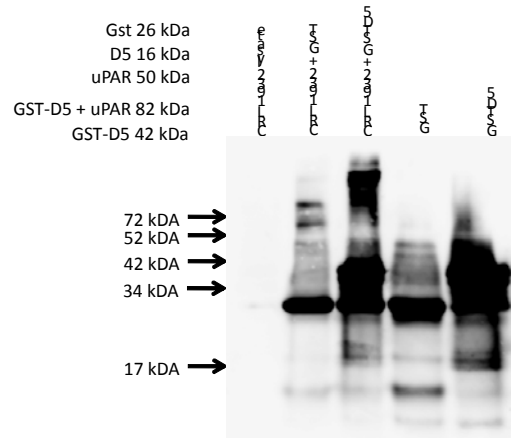

Figure 5. CRL1932 lysate GST pulldown of uPAR. Membrane was incubated with anti-GST antibody at 1:10,000 dilution for 1 hr.

Further experiments to verify binding include competitive binding assays with non-labeled D5, immunofluorescent microscopy, overexpressing uPAR in HEK293 cells, and knocking down uPAR via siRNA. Pulldown experiments will be repeated in cells with knocked down uPAR to verify that the binding is due to D5-uPAR interaction.

2.b. Assess anti-proliferative effects of D5a-nanoparticles.

Task 2.4. Assess anti-proliferative effects of D5a-nanoparticles.

D5 Toxicity. To determine the anti-proliferative effects of D5 conjugated to CNTs, the anti-proliferative effect of D5 alone must first be determined. 6000 HUVEC, CRL1932 and RENCA cells were plated, starved and incubated with recombinant D5, HKa (cleaved high-molecular-weight kininogen), or staurosporine (negative control). HUVECs were treated for 24 hrs. and CRL1932s were treated for 48 hrs. 5 wells were used for each treament group. Dose-dependent decreases in viability (determined by MTT assay) were seen in both HUVEC and CRL1932 cell incubated with D5. (**Figures 6 and 7**) At 3000 HUVEC cells/well, up to 90% of proliferation was inhibited with 50 and 100 nM HKa treatment. [2] Another study found similar inhibition at 50 nM HKa. [3] Our studies have found ~50% growth inhibition in the cell lines studied at 50 nM HKa and slightly greater inhibition at 100 nM. See Supporting Figures for additional data.

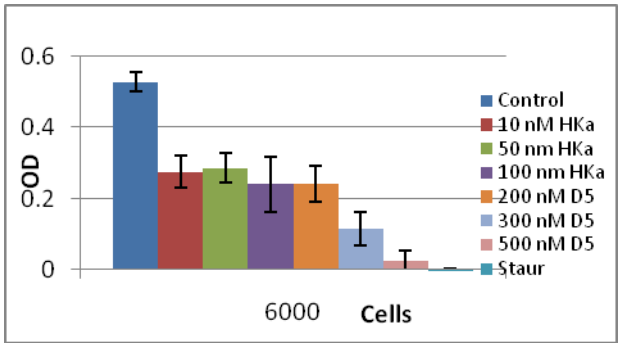

Figure 6. MTT assay of HUVECs incubated with HKa or D5 for 24 hrs.

CNT Toxicity. To measure the inherent toxicity of D5-conjugated CNTs, we also tested the cytotoxicity of CNTs prior to any conjugation. HUVECs and CRL1932 cells were incubated with increasing concentrations of pristine (unmodified), carboxylated (COOH functionalized), and amidated (NH2 functionalized) CNTs for 24. hrs. Clonogenic survival was assessed 7-10 days after CNT incubation after plating 200-300 cells/well. Similar trends were seen using all three types of CNTs in the two cell lines studied. (**Figure 7**) Currently, RENCAs have been treated with pristine and NH2 CNTs. The overall toxicity trends reflect previous work performed in our lab using RENCAs. (**Figure8**) CNT toxicity will be determined in normal primary renal epithelial cells as well.

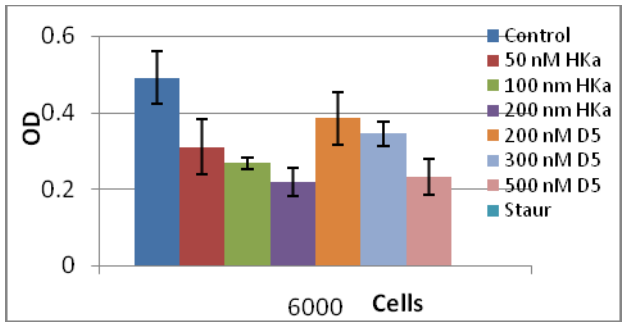

Figure 7. MTT assay of CRL1932 cells incubated with HKa or D5 for 24 hrs.

Thermoablative properties of D5a-nanoparticles.

Task 2.5. Assess thermal ablative properties of D5a-nanoparticles.

CNTs were heated in solution (PBS) without cells to assess their heating properties. 300 ul of increasing concentrations of CNTs were aliquoted in triplicate into 48 well plates. Single-wall CNTs (SWNTs) and multi-wall CNTs (MWNTs) were tested initially. (**Figure 9**) Each well was heated for 30 sec. at 3 W with a 1064 nm YAG laser. Temperature readings were obtained using a probe thermometer. When the experiment was repeated using just MWNTs, the temperature increase plateaued at 100 ug/ml. (**Figure 10**) These findings are consistent with work by others and previous experiments in our lab. [4]

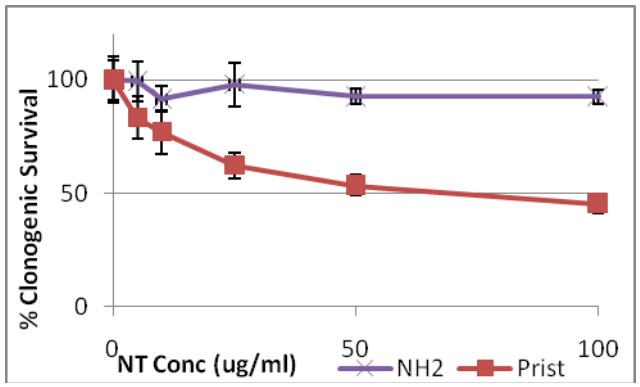

Figure 8. Clonogenic survival of RENCA cells treated with increasing concentrations of MWCNTs.

Cell Survival upon Heating. To assess the effect of CNT heating on cell survival, RENCA and CRL1932 cell lines were heated for 30 or 45 sec. at 3 W laser power with 100 ug/ml pristine CNTs. After heating, cells were plated at 300 cells/well and additionally 3000 cells/well in treated groups. In both cell lines, heating alone and CNT treatment alone results in ~10-20% cell killing, while heating with CNTs results in almost no surviving colonies. (**Figure 11**), Heating and toxicity experiments will be repeated for all cell lines using conjugated CNTs.

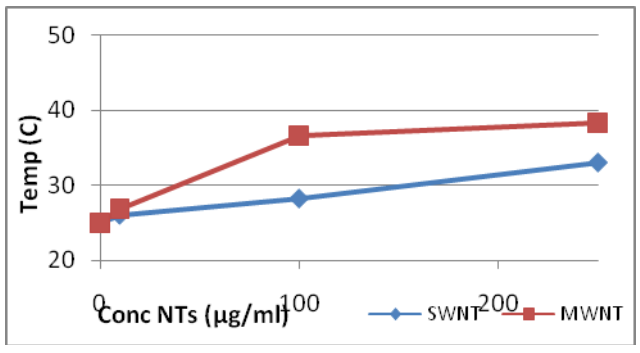

Figure 9. Heating curves of SWNTs and MWNTs after heating with 3 W for 30 sec.

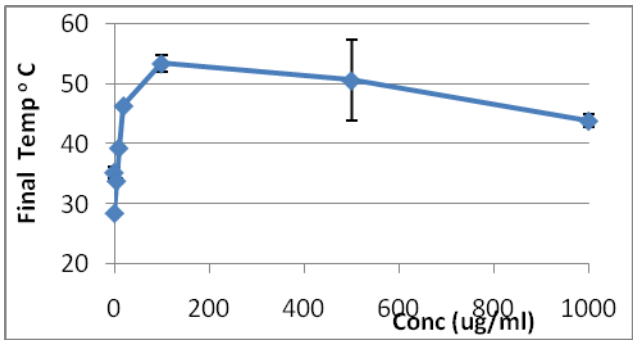

Figure 10. Heating curves of MWNTs after heating with 3 W for 30 sec.

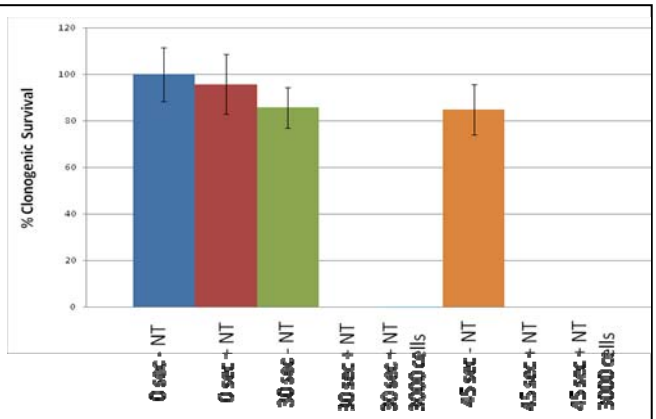

Figure 11. Clonogenic survival of CRL1932 cells after MWNT treatment and heating with 3 W for 30 sec.

Specific Aim 3. Examine accumulation and anti-tumor effect of particles in mice. These experiments are not scheduled to begin until the end of year 2 of this proposal. However, the predoctoral student has practiced establishing xenografts in mice using PC-3 prostate cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

- Identified differences in uPAR expression in normal, cancer and endothelial cells.
- Produced recombinant GST-D5 for conjugation with nanotubes
- Observed that GST-D5 binds to uPAR.
- Demonstrated dose-dependent toxicity of D5 to endothelial cells and renal cancer cell lines.
- Demonstrated that CNTs are toxic to cancer and endothelial cells.
- Demonstrated successful thermal ablation of cultured cells exposed to nanotubes following near-infrared radiation.

REPORTABLE OUTCOMES

The trainee has attended several meetings designed to enhance his training with regard to nanotechnology and use of nanotechnology in anti-cancer therapy. In particular, he attended the Nano Conference in January 2011 sponsored by the American Association for Cancer Research. He also attended the NCL Lessons Learned Workshop sponsored by the National Cancer Institute NanoCharacterization Laboratory in June 2011. He has made two formal presentations of his research to the scientific community. One was at Wake Forest University and a second at was at Wistar Institute in Pennsylvania. He has also successfully passed his preliminary examination and been formally admitted to candidacy.

CONCLUSION

We have shown increased protein expression of uPAR in target cell lines compared to normal cells. This is important because uPAR will be used as a target to attract conjugated CNTs to the tumor site. Treatment of cell lines with D5 induces similar toxicity as HKa, albeit at higher levels. CNTs were shown to be inherently toxic to cell lines. Functionalization appeared to have a small effect on decreasing toxicity. Knowing these two aspects of toxicity will allow for relevant comparisons of toxicity when conjugated CNTs are developed. Heating cells with CNTs alone proved to be quite toxic. Again, this will be used for comparison when cells are heated with conjugated CNTs. It will be important to test all of these experiments using normal cells. Clonogenic survival upon CNT incubation was unsuccessful as these cells did not form colonies well. Another technique will have to be used to assess cytoxicity in non-tumor cells since CNTs interfere with the absorbance wavelength of MTT assays, and clonogenic survival assays do not work in these cells. The Cell Death Detection ELISA kit from Roche is read at a wavelength that should be outside CNT absorbance. Upon conjugation and *in vitro* testing of conjugated CNTs, we will be able to begin testing using animal models.

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